Short Communications

A New Fluorescence Method for Detection and Possible Quantitative Assay of some Catecholamine and Tryptamine Derivatives on Paper

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The reaction between catecholamines and formaldehyde in the presence of dry protein films has been discussed by Falck, Hillarp, Thieme & Torp (1962) and its nature has been elucidated by Corrodi & Hillarp (1963, 1964).

These authors have shown that the initial condensation of substituted phenylethylamines (I) with formaldehyde to form tetrahydroisoquinolines (II) takes place readily in solution if an activating group is present in the *meta* position, but that the dehydrogenation of (II) to the fluorescent dihydroisoquinolines (III) by formaldehyde only takes place in a dry film, which can be of protein, as in tissue sections, or of certain amino acids or dipeptides.

We have now used this reaction as the basis for a convenient and sensitive method of detecting catecholamines and their metabolites on paper, and in some cases the reaction products have been eluted and measured quantitatively. In conjunction with other methods, such as ethylenediamine condensation, which depend on different characteristics of the molecule, this should be a valuable tool for identifying these metabolites. Some tryptamines also give fluorescent products.

For test purposes 0.01–10 μg . of the compound was spotted on Whatman no. 1 paper and dried. Optimum conditions for developing the fluorescence were as follows: the papers were sprayed with 5% (w/v) glycine adjusted to pH 3.0 with HCl, and oven-dried at 80° before being suspended in a

sealed Kilner jar (3l.) containing paraformaldehyde (20g.) moistened with water (6ml.). The jar was then heated in an oven at 80° for 3 hr. and the papers were viewed under u.v. light of wavelength 360 $\text{m}\mu$. The fluorescent compounds were eluted from the paper with 0.1N-HCl and their spectral characteristics measured in an Aminco-Bowman spectrofluorimeter. These eluates were stable for several days at 0–5°. Dihydroxyphenethylamine (dopamine) and dimethoxyphenethylamine were tested at various loadings and the fluorescence was found to be linear over the range 0.2–4 μg . at least. Table 1 lists the compounds tested and shows the minimum detectable amount, the colour of the spot and in some cases the principal activation and fluorescence maxima of the eluate. Tyramine, dimethoxyphenylacetic acid, histamine, histidine and ergothioneine did not react.

Numerous variations in the conditions were tried before the optimum was arrived at. Glycine was the most convenient 'carrier' reagent: glycylglycine was as effective, but sucrose, starch, phenylalanine and rat-brain homogenate were less effective, as were oxidizing agents, e.g. hydrogen peroxide, periodic acid or ferricyanide. The pH of the glycine treatment was also important, the fluorescence yield

Table 1. *Fluorescence characteristics of catecholamines and other compounds after glycine-formaldehyde treatment.*

Details are given in the text.

Compound	Minimum detectable amount ($\mu\text{g.}$)	Colour	$\lambda_{\text{max.}}$ ($\text{m}\mu$) (uncorrected)	
			Activation	Fluorescence
Noradrenaline	0.03	Greenish yellow	430	540
Adrenaline	1	Dull blue-grey		
Dopamine (3,4-dihydroxyphenethylamine)	0.03	Greenish yellow	430	525
Dopa (3,4-dihydroxyphenylalanine)	0.1	Buff-orange	430	530
Metadrenaline	10	Dull olive green		
Normetadrenaline	1	Primrose		
4-Hydroxy-3-methoxyphenethylamine	0.03	Bright yellow	440	520
3-Hydroxy-4-methoxyphenethylamine	0.03	Bright ice blue	360	465
3,4-Dimethoxyphenethylamine	0.03	Bright ice blue	360	470
Mescaline	1	Dull yellow		
Octopamine	10	Dull blue-violet		
α -Methyl- <i>m</i> -tyrosine	10	Blue-violet		
4-Hydroxy-3-methoxymandelic acid	10	Apple green		
Tryptamine	0.03	Greenish yellow		
5-Hydroxytryptamine	0.03	Yellow-brown		
Tryptophan	1	Light blue		
Melatonin	1	Yellow-brown		
Bufotenine	1	Dull primrose		
Kynurenine	1	Pale blue-grey		
3-Hydroxykynurenine	1	Buff		
Xanthurenic acid	1	Blue-grey		
Kynurenic acid	1	Light blue		
Anthranilic acid	1	Royal blue		

falling off markedly above pH 3.0. Drying the paper thoroughly before formaldehyde treatment was important, but some moisture in the formaldehyde was necessary though the amount was not critical. The amount of heat required for the formaldehyde reaction was very variable: some compounds, e.g. noradrenaline and dopamine, reacted strongly in 30 min. at 50°, whereas others, e.g. dimethoxyphenethylamine, required much longer. Even at 80° for 3 hr. adrenaline did not react strongly, but further heating offered no advantage.

The differing behaviour of the methoxylated phenethylamines is interesting. Whereas compound (IV) gave a yellow fluorescent product [$\lambda_{\text{max.}}$ at 440 $\text{m}\mu$ (activation) and at 520 $\text{m}\mu$ (fluorescence) in acid solution] similar to that from dopamine, both compound (V) and compound (VI) gave a blue product [$\lambda_{\text{max.}}$ at 360 $\text{m}\mu$ (activation) and at 470 $\text{m}\mu$ (fluorescence) in acid solution]. According to Corrodi & Hillarp (1964) dopamine and compound (VI) give products with the same spectrum below pH 5 (the spectrum that we also obtained from compound VI), but above pH 5 differences appear owing to the formation of the quinone (VII) from the dopamine products. Even above pH 5 they did not observe any fluorescent emission higher than 480 $\text{m}\mu$, and, since our compounds show their

differences in acid solution, it seems likely that our conditions result in further oxidation of the dihydroxy compound with the formation of a more stable quinone form fluorescing at 530 $\text{m}\mu$. Further, since compound (V) behaved like compound (VI), where no quinone formation can take place, and dopamine behaved like compound (IV), the quinone must be of the type (VIII) rather than (VII). (Compounds IV and V were obtained from Calbiochem, Lucerne, Switzerland, and their structures have been confirmed by Mr D. Greatbanks by nuclear-magnetic-resonance spectroscopy in alkaline solution: the O^- at position 3 produces a shift in the protons at positions 2 and 6 that does not occur when O^- is in position 4.) Authentic specimens of 6,7-dihydroxy- and 6,7-dimethoxy-3,4-dihydroisoquinolines spotted on paper showed the same blue fluorescence as the product from compound (VI), but after glycine-formaldehyde treatment of the papers the dihydroxy compound altered to give the yellow fluorescence of the product from dopamine, whereas the dimethoxy compound remained unchanged. This is further evidence that some additional reaction has taken place.

The tryptamine fluorescence appeared to be due to a mixture of compounds that were eluted from the paper at different rates, giving rise to variable

spectra in solution. One of these may be the 3,4-dihydro- β -carboline (IX), suggested by Corrodi & Hillarp (1963), which has a yellow fluorescence; the other, and more readily eluted, component has a lower fluorescence maximum and has not been identified.

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The Rate-Limiting Step in the Oxidation of Palmitate or Palmitoyl-Coenzyme A by Rat-Liver Mitochondria

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It is generally agreed that the rate of β -oxidation of fatty acids in intact mammals is largely controlled by the availability of fatty acids to the tissues concerned (Fritz, 1961), but there is little information or agreement on the rate-limiting steps of fatty acid oxidation in systems supplied with non-limiting concentrations of fatty acids. Reactions catalysed by acyl-CoA synthetase (EC 6.2.1.3; Krebs, 1964; Bode & Klingenberg, 1964) and acyl-CoA dehydrogenase (EC 1.3.2.2; Bunyan & Greenbaum, 1965) have both been described as rate-limiting for fatty acid oxidation.

The low concentration of intermediates of β -oxidation in rat-liver mitochondria oxidizing palmitoylcarnitine (Garland, Shepherd & Yates, 1965) is consistent with organization of the enzymes concerned into a complex, and this possibility is further supported by studies that demonstrate a close relationship between the enzymes of β -oxidation and a barrier or membrane that requires carnitine for acyl transfer (D. W. Yates & P. B. Garland, unpublished work). The activities of enzymes extracted from mitochondria may not represent their normal intramitochondrial activities, and the studies reported below refer to intact mitochondria.

Methods. Preparation of mitochondria and substrates, measurement of mitochondrial oxygen uptake and fluorimetric assay of CoASH with oxoglutarate dehydrogenase (EC 1.2.4.2) (reaction 2) have been described by Garland *et al.* (1965). All mitochondrial incubations were made at 25° in a final volume of 2.1 ml. containing KCl (80 mM), tris-chloride buffer, pH 7.2 (20 mM), $MgCl_2$ (2 mM), EDTA (1 mM) and mitochondrial protein (1–3 mg./

ml.). Other additions are described below. The carnitine-palmitoyltransferase activity (EC 2.3.1.–; Norum, 1964) of intact mitochondria was assayed by coupling carnitine-dependent CoASH release from palmitoyl-CoA (reaction 1) with the fluorimetric CoASH assay:

Further additions to the incubation mixture for this assay were rotenone (0.4 $\mu\text{g./ml.}$), KCN (0.1 mM), 2-oxoglutarate (1.0 mM), NAD (1.0 mM), oxoglutarate dehydrogenase [20 $\mu\text{g.}$ (0.06 unit)/ml.], palmitoyl-CoA (40 μM) and DL-carnitine (2.5 mM). Carnitine was added last, and the concentration used was that which gave maximal stimulation of palmitate or palmitoyl-CoA oxidation in respiring mitochondria.

Experimental design. Under suitable conditions (Shepherd, Yates & Garland, 1965) the oxidation of palmitate, palmitoyl-CoA or palmitoylcarnitine by rat-liver mitochondria results in a stoicheiometric conversion into acetoacetate or citrate, and the rate of palmitate or palmitoyl-CoA oxidation (as $\mu\text{moles of } C_{16:0} \text{ oxidized/min./mg. of mitochondrial protein}$) can be calculated from the observed oxygen uptake rate after correction for the oxidation of endogenous substrates. Under the conditions used, palmitate or palmitoyl-CoA oxidation is absolutely carnitine-dependent, and the correction for oxidation of endogenous substrates is unlikely to include any fatty acid oxidation. The reactions resulting in palmitate oxidation may be